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# Pattern of nucleotide substitution and divergence of prophenoloxidase in decapods

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**Abstract**

Despite the unprecedented development in identification and characterization of prophenoloxidase (proPO) in commercially important decapods, little is known about the evolutionary relationship, rate of amino acid replacement and differential selection pressures operating on proPO of different species of decapods. Here we report the evolutionary relationship among these nine decapod species based on proPO gene and types of selective pressures operating on proPO codon sites. Our analyses revealed that all the nine decapod species shared a common ancestor. The mean percentage sequence divergence at proPO gene was $34.4 \pm 0.6\%$. Pairwise estimates of nonsynonymous to synonymous ratio ($\omega$) for *Homarus americanus–H. gammarus* is greater than one, therefore indicating adaptive evolution (functional diversification) of proPO in these two species. In contrast, strong purifying selection ($\omega < 1$) was observed in all other species pairs. However, phylogenetically closely related decapods revealed relatively higher $\omega$ value ($\omega = 0.15 \pm 0.3$) than the distantly related species pairs ($\omega = 0.0075 \pm 0.005$). These discrepancies could be due to higher fixation probability of beneficial mutation in closely related species. Maximum likelihood-based codon substitution analyses revealed a strong purifying selection operating on most of the codon sites, therefore suggesting proPO is functionally constrained (purifying selection). Codon substitution analyses have also revealed the evidence of strong purifying selection in haemocyanin subunits of decapods.


*Keywords:* Decapod; Prophenoloxidase; Haemocyanin; Codon substitution; Nonsynonymous; Synonymous; Genetic distance

## 1. Introduction

The prophenoloxidase [proPO: zymogen of phenol oxidase (PO; monophenol, L-dopa: oxygen oxidoreductase, EC1.14.18.1)] plays crucial role in innate immunity of invertebrates, for example, sclerotization of arthropod cuticle, pigmentation, wound healing and humoral immune defence [1]. Due to the lack of adaptive immunity in invertebrates, the focus has been towards enhancing innate immune system in many of the cultured species, especially crustaceans [2–7]. Despite the unprecedented development in identification and characterization of proPO (e.g. [2–7]), little is known about the evolutionary relationship of proPO present in different species of arthropods, especially in decapods

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except the fact that phylogenetically it belongs to the arthropod haemocyanin super-family [8–10]. Although based on its activity proPO is similar in all species, the expression of proPO is species specific [2–7] and influenced by both intrinsic (cellular) and extrinsic environmental factors [11]. Based on this, one might expect that differential selective pressure is operating on the protein-coding region of proPO during the evolutionary time scale. The selective pressure in the protein-coding gene can be measured by comparing the number of nonsynonymous substitutions per nonsynonymous site ($d_N$) with the number of synonymous substitutions per synonymous site ($d_S$) [12]. The mutational change that resulted in a change of amino acid is known as nonsynonymous substitution, whereas in synonymous substitution, amino acid remains unchanged even though there is a change in nucleotide. There are three different types of selective pressures that can be detected from $d_N$ and $d_S$ ratio (hereafter referred as $\omega$). If the protein-coding gene is functionally constrained, that is, if most nonsynonymous mutations are deleterious, then the rate of nonsynonymous change will be lower than neutral rate resulting in $\omega < 1$ and the gene is subject to be under strong purifying selection [13]. If nonsynonymous mutations are beneficial, average rate of nonsynonymous changes is expected to be higher than neutral rate, resulting in $\omega > 1$, indicating functional diversification of the gene and subject to positive selection. The evolution of pseudogenes is attributed to the lack of functional constraint on the protein coding genes and is referred to as neutral evolution ($\omega = 1$).

Many of the adaptive and innate immunogenic genes are reported to be under the influence of positive selection (e.g. [14]). It could be possible that selective pressure might also be operating on the entire coding region of proPO. However, if the entire coding region of proPO is not under the influence of positive selection, it is possible that positive selection might be operating on a few codon sites of proPO. The aim of the present study is to investigate the type of selective pressure operating on codon sites of proPO of nine commercially important decapods using maximum likelihood-based codon substitution analyses [15]. From the proPO nucleotide sequence data of these nine decapods, the evolutionary relationship and degree of genetic divergence among these decapods are also estimated using maximum likelihood, Bayesian, maximum parsimony and distance based phylogenetic methods [16–18].

## 2. Materials and methods

### 2.1. Phylogenetic analyses

#### 2.1.1. Amino acid phylogeny

To infer evolutionary relationship between arthropod proPO and haemocyanin, we reconstructed maximum likelihood (ML), Bayesian inference (BI) and neighbour joining (NJ) phylogenies based on the amino acid sequence data. A total of 55 amino acid sequences representing Crustacea and Insecta haemocyanin and proPO genes were retrieved from the GenBank ([3–5,19–48], Table 1). All the amino acid sequences were aligned using DAMBE ver. 4.5.2 [49,50]. The unrooted ML and BI amino acid phylogenies were reconstructed using PHYML ver. 2.4.4 [51] and MrBayes 3.04 [16] programs, respectively. Amino acid ML analyses were performed using an input tree generated by BIONJ [52]. The JTT model of sequence evolution was used in both ML and BI analysis. NJ tree was performed with Poisson correction amino acid model using MEGA 2.0 [17]. The nodal support for NJ tree was estimated with 10,000 bootstrap replicates using MEGA 2.0 [17]. PHYML bootstrap trees were constructed using the same parameters as the ML trees. The amino acid BI analyses were performed by running four simultaneous chains for $1.5 \times 10^6$ generations and sampling every 1000 generations. All trees below the observed stationary level were discarded, resulting in a "burn-in" of 15,000 generations. As noted above, the fluctuating value of log likelihood was plotted in Tracer ver. 1.3.1 [53] to verify that convergence was reached. The 50% majority-rule consensus tree was used to calculate the posterior probabilities for each node.

#### 2.1.2. proPO nucleotide phylogeny

A total of 11 published proPO and 2 haemocyanin nucleotide sequences representing nine species of decapods were obtained from the GenBank ([3,5,19,20,30,45], Table 1). As described by Burmester [8], haemocyanin sequences were used as out-group in proPO phylogeny. After alignment of all the 11 proPO sequences, a 2085 base pairs (bp) sequence length was produced. When the out-group sequences were included, the total length of all 13 species became 2178 bp as there were several inserts in the haemocyanin gene. All sequences were aligned using MacClade 4.03 [54] and DAMBE ver. 4.5.2 [49,50].